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Observations on the Phagocytosis and Elimination of Carmine Particles Injected into the Abdominal Musculature of the White Shrimp, *Penaeus setiferus*[1]

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White shrimp, *Penaeus setiferus,* were injected in the abdominal musculature with 0.03 ml of a 1.4% carmine-saline solution and were kept at a temperature of 26°–28°C and a salinity of 23–26‰. Tissue samples were taken at hourly intervals of 1, 6, 12, 18, 24, 30, 36, 42, and 72 hr and at 4, 6, 8, 10, 12, 16, 19, and 33 days post-injection and were examined histologically to determine the sites of phagocytosis and elimination of foreign particulate matter. Within 1 hr post-injection, extracellular clumps of the carmine particles were formed in the hemolymph. These clumps had been invaded by hemocytes at 18 hr, but they persisted throughout the study. Phagocytosis of the particles was accomplished by hemocytes circulating in the hemolymph and by fixed phagocytes in the gill, heart, loose connective tissue, and blood sinusoids in the abdomen. The fate of some phagocytized carmine was elimination by the migration of hemocytes through the epithelium of the gills, gut, hepatopancreas, and through the extremities of the pereiopods and the pleopods. Encapsulations or brown nodules were formed in the musculature of the pereiopods around necrotic hemocytes that had phagocytized carmine. A large blister or cyst filled with carmine was formed in the gill cover of one specimen. The observation of carmine particles at 33 days post-injection indicates a slow clearance rate of large amounts of abiotic particulate matter in penaeid shrimp.

INTRODUCTION

Investigations into methods of marking commercially important penaeid shrimp for population dynamics studies have, historically, included either injecting, feeding, or immersing the shrimp into various biological stains, dyes, and ink (Neal, 1969). The objective of these investigations was to find a satisfactory technique for mark-recapture experiments to determine such parameters as growth, mortality, and migrations of shrimp populations. The success or failure of a stain being injected as a marker was gauged by the percentage mortality attributable to the technique, duration of the marker, and ease of visual recognition. The value of a particular stain as a marker depends primarily upon the rate of cellular uptake and elimination of the particles of stain from the shrimp. These stains and dyes provide an ideal model for the study of the cellular defense mechanism in penaeid shrimp.

In invertebrates in general, the components of this defense mechanism when reacting to a foreign particulate material are: coagulation, accumulation, phagocytosis, and subsequent elimination (Cheng et al., 1968). As early as 1905, Cuénot described the cellular phagocytic activity of a number of decapod crustaceans that included several species of prawn and the lobster *Homarus vulgaris.* More recently, the volumes of documented reports of this

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response in insects was reviewed (Salt, 1970) while this response in invertebrates other than insects was reviewed by Sparks (1972). Further, additional information on the phagocytosis of foreign material in the cockroach (Ryan and Nicholas, 1972) and the oyster (Foley and Cheng, 1972) have been recorded. This paper presents observations on the cellular response of the marine decapod crustacean, *Penaeus setiferus* (white shrimp), to the injection of carmine particles into the abdominal musculature. The gross morphological nomenclature used here is based on that published for the white shrimp (Young, 1959), while the histological terminology is taken from that of Dennell (1960) and Salt (1970).

Materials and Methods

The shrimps used in this study were acclimated to and maintained at a temperature of 26°C–28°C and a salinity of 23–26‰. During the 48-hr period of acclimation all shrimp that appeared to be injured or unhealthy were removed. Test animals were injected using tuberculin syringes with 27-gauge needles between the fifth and sixth abdominal somites with 0.03 ml of a suspension containing 1 g of carmine in 69 ml (1.4% solution) of sterile seawater solution. In their work on the sponge, Cheng et al. (1968) injected 0.2 ml of a 1:10 seawater suspension of carmine. This same volume and concentration produced 100% mortalities within 30 min when injected into test shrimp ranging in total length (tip of rostrum to tip of telson) from 110 to 140 mm. Tolerance tests indicated that 0.03 ml of a 1.4% carmine-saline suspension was the maximum that could be injected into shrimp ranging in total length from 90 to 150 mm with little or no mortality.

Samples for histological examination were taken at hourly intervals of 1, 6, 12, 18, 24, 30, 36, 42 and 72 hr; and at 4, 6, 8, 10, 12, 16, 19, and 33 days post-injection. A section of tissue from the site of injection as well as the entire cephalothorax was taken from each sample and fixed in 10% buffered formalin. The fixative was changed after 24 hr. Tissue specimens were then decalcified for 8 days using a modification of the formic acid method of Evans and Krajian (Krajian, 1940), and after decalcification, tissues were washed in tap water for 4–6 hr. The tissue specimens were then routinely processed and stained with Harris' hematoxylin. Eosin was not used as a counterstain to avoid masking the carmine particles.

Results

Gross Appearance

Immediately after injection, the carmine particles were dispersed throughout the body of the shrimp, imparting to the shrimp a bright red coloration, but within 1 hr the particles were being accumulated in the dorsal abdominal artery, ventral abdominal vein, heart, and gills. Carmine was visible externally only in the gills, heart, and at the site of injection by 30 hr. One test animal still had carmine particles visible at the site of injection and in the gill filaments at 33 days post-injection.

Histological Observations

Carmine particles at the site of injection appeared to be clumped together forming tightly packed extracellular masses at 1 hr post-injection (Fig. 1a). At 18 hr, however, the clumps were infiltrated with numerous hemocytes that contained carmine, some forming partial occlusions of blood vessels (Fig. 1b). Carmine particles were observed during the first 30 hr post-injection either free or within phagocytes in all shrimp tissue with the exception of the ovarian, optic, and nerve tissues. Sites of accumulation thereafter appeared to be within the gill filaments and in the heart (Fig. 1c), where free particles and hemocytic aggregations were observed in the blood sinuses.

Hemocytes were first observed in great numbers in the area of injection at 30 hr post-injection (Fig. 1d) and were actively

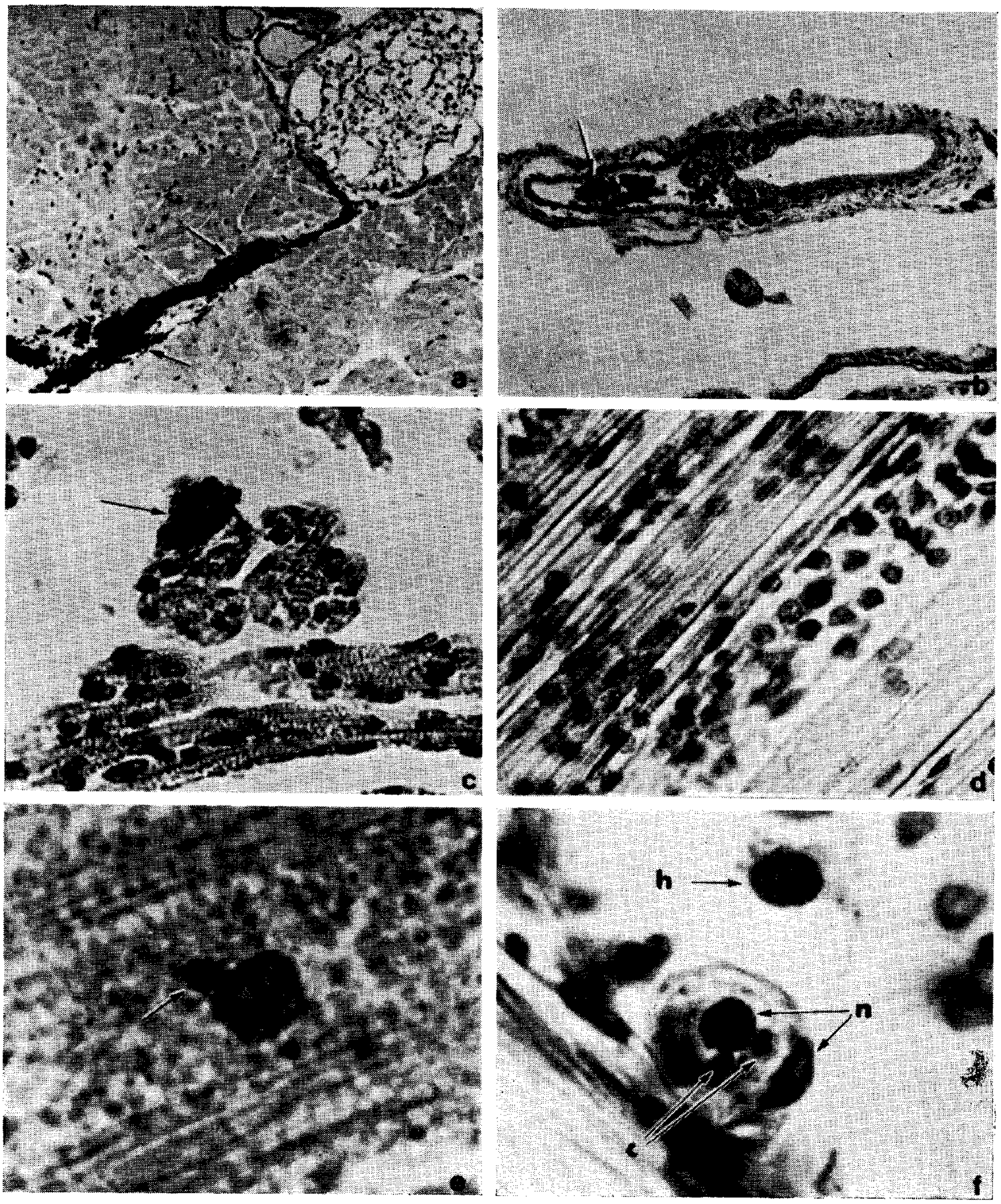

FIG. 1. (a) Clots of carmine formed in the area of injection (arrows); 1 hr, ×108. (b) Hemocytic aggregation in dorsal abdominal artery forming partial occlusion (arrow); 18 hr, ×189. (c) Hemocytic aggregation in the heart; 6 hr, ×171. (d) Infiltration of hemocytes into area of injection; 30 hr, ×690. (e) Hemocyte with phagocytized carmine; 30 hr, ×1740. (f) Hemocytic "Clump," in area of injection containing two nuclei (n = nuclei, c = carmine, h = free hemocyte); 72 hr, ×1305.

phagocytizing the carmine particles (Fig. 1e). Peculiar clumps of hemocytes were being formed in the area of injection at 72 hr (Fig. 1f). These hemocytic clumps usually contained 2 to 3 nuclei and possessed phagocytized carmine. Large wandering hematogenous cells had also appeared in the area of injection at 42 hr and were engaged in phagocytizing the carmine particles (Fig. 2a).

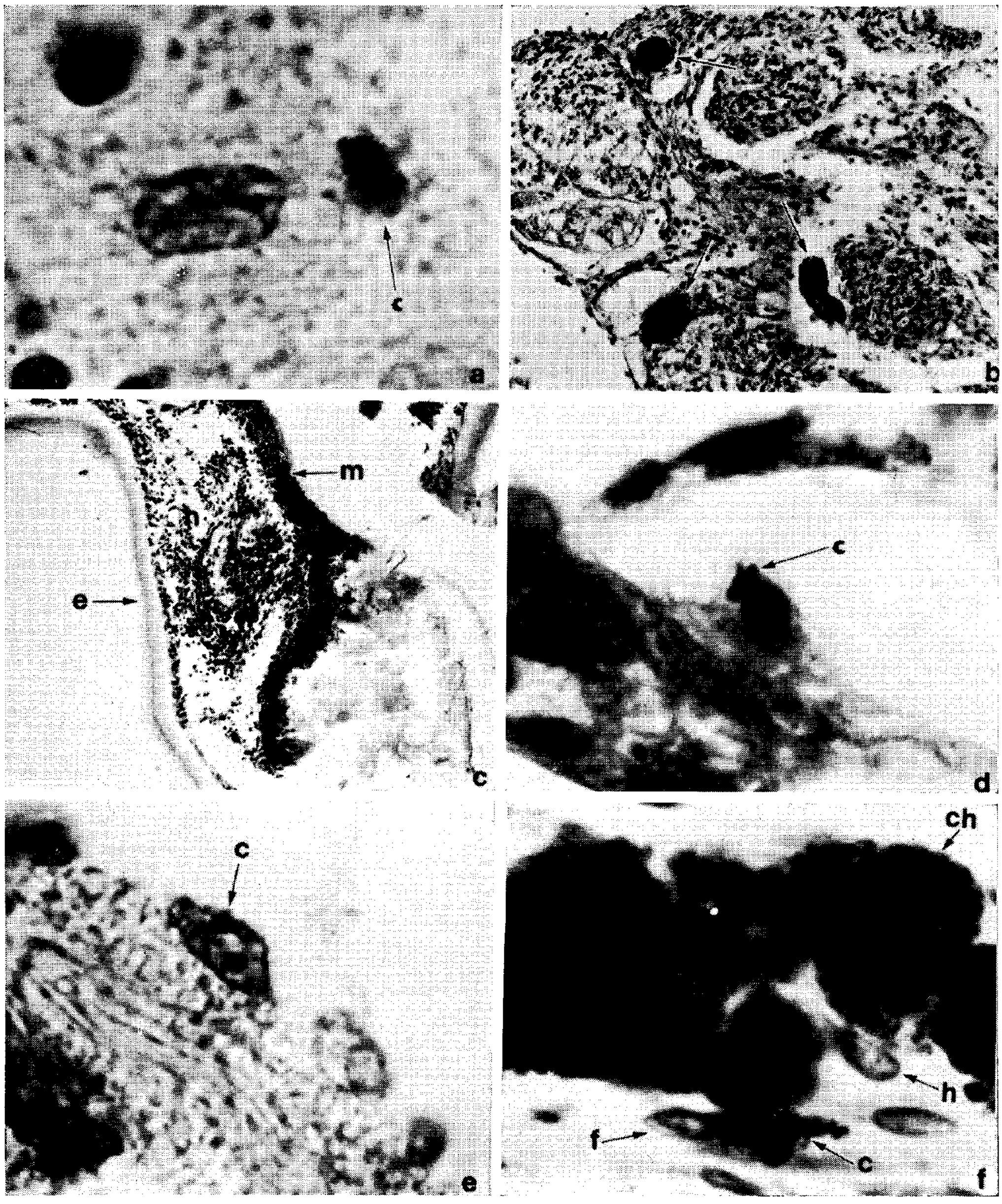

FIG. 2. (a) A phagocyte containing carmine (arrow) in the musculature at the site of injection; 30 hr, ×2052. (b) Brown nodules of encapsulation (arrows) in pereiopod; 96 hr, ×144. (c) Cyst containing a large number of hemocytes with carmine in the gill cover (e = external cuticle, h = hemocytes, m = melanized internal wall); 42 hr, ×90. (d) Fixed phagocyte in gill filament (c = phagocytized carmine); 30 hr, ×1305. (e) Fixed phagocyte in blood sinus of heart (c = phagocytized carmine); 30 hr, ×1305. (f) Fixed phagocyte in abdominal blood sinusoid (f = fixed phagocyte, c = carmine, H = free hemocyte, ch = carmine phagocytized by hemocyte); 30 hr, ×1305.

Brown nodules had appeared at 4 days post-injection in the connective tissue of the pereiopods (Fig. 2b). The encapsulated material appeared to be necrotic hemocytes that contained phagocytized carmine. No free particles were observed within the nodules. Also, a large encapsulation or "cyst" was observed in the gill cover of one

test shrimp at 42 hr (Fig. 2c). The cyst appeared as a pocket of necrotic hemocytes and carmine particles in the connective tissue layer on the gill side of the gill cover and protruded into the gill cavity. The outer epidermis and cuticle appeared normal.

The blood vessels, Chambers' sinuses, and sinusoids that were examined appeared to be lined with a network of cells that had begun to phagocytize carmine at 6 hr post-injection. Phagosomes containing carmine in these cells persisted throughout the study. These phagocytic cells were observed in the linings of the blood sinusoids of gill filaments (Fig. 2d), in the heart (Fig. 2e), and in the abdomen (Fig. 2f).

The loose connective tissue just basal to the epidermis in the posteriodorsal area of the cephalothorax also contained many large round or oval fixed phagocytic cells (Fig. 3a). The cells had a small, acentric nucleus and extensive lightly basophilic cytoplasm. The cytoplasm contained numerous phagosomes at 18 hr, and approximately 25% of these cells had small amounts of carmine included throughout the study.

The gills contained carmine at 1 hr, either free in the hemolymph as clumps or included in phagocytes that lined the lumen. At 6 days, the extracellular particles and individual hemocytes had been replaced by hemocytic aggregations occurring most frequently in the distal terminus of the gill filaments.

Free carmine particles and hemocytes that had phagocytized carmine occurred in the hepatopancreas at 18 hr (Fig. 3b); however, no carmine was observed in the lumen of any of the acini. Hemocytes that had phagocytized carmine were also observed migrating through the midgut wall at 24 hr post-injection (Fig. 3c). Although hemocytes appeared to be migrating through the epithelium of the gut wall, none were observed there beyond 4 days, nor were any free particles of carmine observed in the gut lumen.

At 72 hr, and, continuing throughout the study, the antennal gland was observed to contain numerous phagocytic hemocytes with carmine particles included (Fig. 3d). The antennal gland is connected to an external pore by a short duct (Young, 1959) that was not seen in the tissue sections examined; however, hemocytes with carmine included may have been eliminated via this duct.

The terminal ampoule, a glandular structure that produces the spermatophore (Young, 1959), is situated above the male gonopore on the sternum. The ampoule was interspersed with numerous hemocytes with phagocytized carmine at 18 hr (Fig. 3e). Although the process was not seen, it appears that the terminal ampoule of the male and the gonopore on the sternum are points of elimination for hemocytes. There were no carmine or hemocytes with phagocytozed carmine observed in the ovarian tissue examined.

The muscle and connective tissue associated with the maxillae, pereiopods, and pleopods was congested with phagocytic hemocytes at 12 hr post-injection. Hemocytes containing carmine were observed adhering to or in close proximity to the setae of the pleopods (Fig. 3f) at 72 hr while many phagocytic hemocytes were situated between epithelial cells of the epidermis in these areas. Although the migration of hemocytes through the body wall was not observed, their probable route of exit was via the cuticular pores from which the setae originate.

Discussion

The process of phagocytosis and elimination in the penaeid shrimp closely resembles that described for insects (Ryan and Nicholas, 1972; Salt, 1970; Werner and Jones, 1969). According to Salt (1970), phagocytosis, nodule formation, encapsulation, and melanization are all part of a general hemocytic response in insects, the process occurring being related to particle size. Although the cells of the hemolymph of

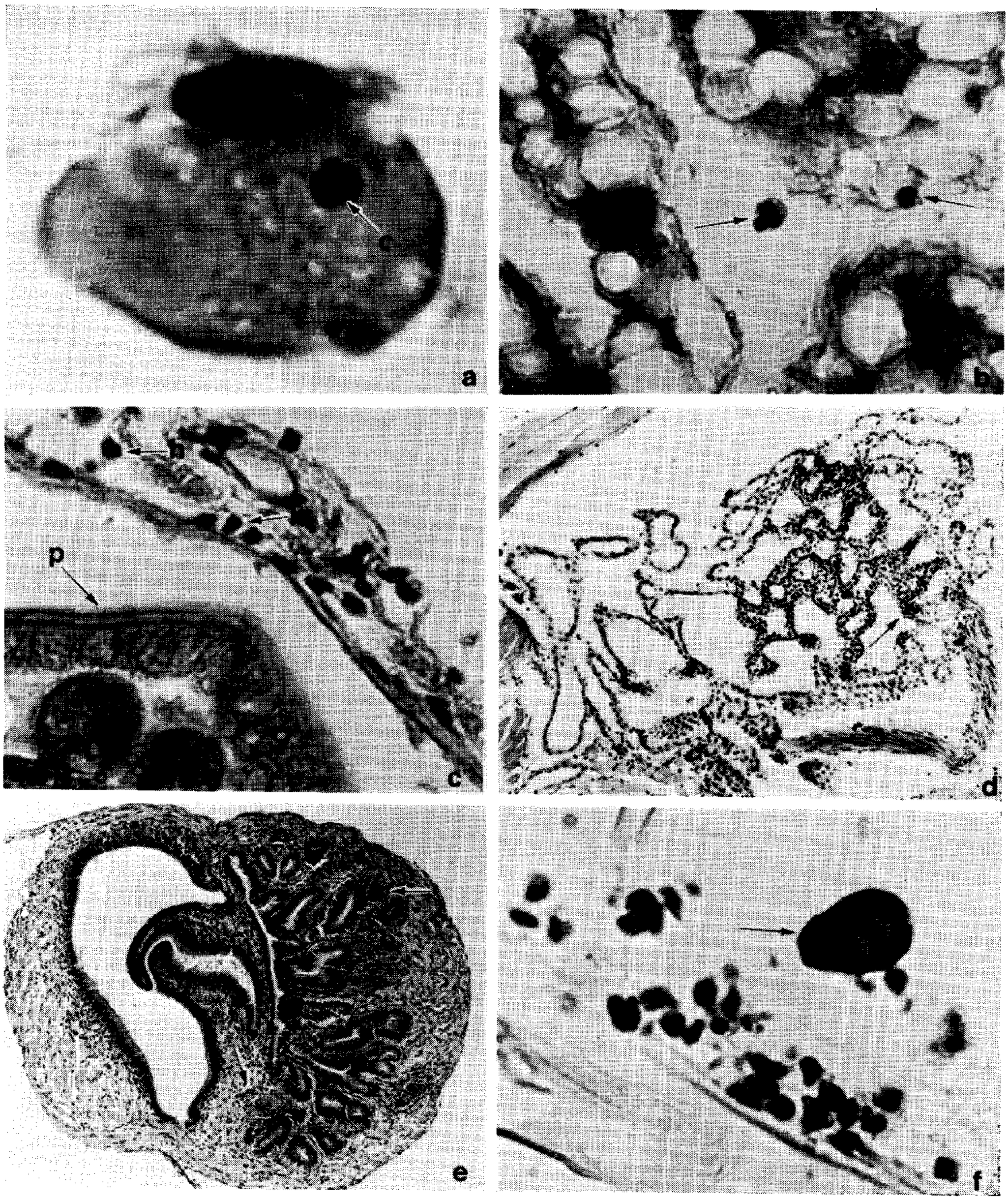

FIG. 3. (a) Fixed phagocyte in subcuticular loose connective tissue of cephalothorax (c = carmine); 30 hr, ×3300. (b) Hemocytes with phagocytized carmine in hepatopancreas (arrows); 18 hr, ×702. (c) Hemocytes migrating through midgut wall. The separation between the epithelium and the wall is an artifact (h = hemocytes, p = nematode parasite in lumen of gut); 24 hr, ×873. (d) Antennal gland with scattered clumps of hemocytes that contain carmine (arrows); 72 hr, ×189. (e) Terminal ampoule with "clumps" of hemocytes containing carmine (arrows); 12 hr, ×144. (f) Hemocytes with phagocytized carmine adhering to or in close proximity to an external setae. Arrow points to be ciliated protozoan; 72 hr, ×693.

shrimp have not been morphologically typed, we do know something of their function. The cellular encapsulation of foreign or necrotic material and the eventual formation of "brown bodies" or nodules have been documented for decapod crustacea by Sindermann (1971) and Bang (1970). The development of brown nodules by hemo-

cytes was described in penaeid shrimp (Fontaine and Lightner, 1973), and their formation was attributed to bacteria introduced into a wound. A question that arises and will eventually be resolved is: Are the hemocytes that phagocytize and those that encapsulate and become melanized morphologically distinct, or, does the same cell type perform both functions depending on particle size?

Histological analogies made between the vertebrate and the invertebrate, despite similarities in structure or function, must be used cautiously to avoid confusing or misleading information. The large hematogenous cells observed migrating into the injection site and phagocytizing carmine and the large "clumps" or multinucleated cells formed by phagocytic hemocytes in shrimp in this study are perhaps analogies of wandering macrophages and foreign body giant cells of vertebrates (Bloom and Fawcett, 1970). Furthermore, the phagocytic cells lining the heart, gill, and abdominal blood sinusoids of shrimp appear to be fixed and may be similar to reticuloendothelium described in insects by Wigglesworth (1970). Salt (1970), however, concluded that the phagocytic cells observed in the pericardium of insects are always hemocytes and that these cells may be seen in other tissues. Cells which had phagocytized carmine were also observed adherent to heart tissue in the cockroach (Ryan and Nicholas, 1972), but the authors surmised that they may have been hemocytes. Stauber (1950) observed ink-laden phagocytes in the lining of the pericardial wall and the outer aspect of the heart of the oyster, but concluded they were migrating blood cells.

In oysters injected with India ink, the chief sites of elimination were the stomach, intestine, rectum, and digestive diverticula (Stauber, 1950), findings which agree with our observations on shrimp. There were no migrating ink-laden phagocytes seen in the epithelia of the external (shell secreting) face of the mantle, the gonoducts, excretory tubules, and very rarely were they seen in the epithelium of the gills which is in direct contrast to our observations on shrimp injected with carmine.

The process of phagocytosis and elimination of foreign material into the sponge (Cheng et al, 1968) is similar to that of shrimp in that fixed and free phagocytes in the sponge engulf the particles and migrate to the external surface and in that extracellular clots of the particles are formed in the sponge mesoglea.

It is of interest to note that the cyst or "blister," which was experimentally induced in the gill cover of a shrimp in this study, has been observed to occur in penaeid shrimp from natural environments. A pink shrimp, *Penaeus duorarum,* which was collected from a shallow bay in Florida and sent to the authors, had numerous large white blisters on both gill covers. Close examination showed the blisters to be congested with spores of the microsporidian, *Thelohania* sp. Relatively few of the spores were seen in the ovarian tissue which this parasite normally invades. Many of the shrimp taken from Galveston Bay, Texas, by commercial bait dealers have also been observed to contain numerous black cysts or blisters on the gill covers (Baxter pers. commun.). The experimentally induced cyst and those occurring naturally indicate the loose connective tissue of the gill cover of shrimp may be used as a reservoir for necrotic or foreign material phagocytized by the hemocytes prior to elimination.

Phagocytosis and elimination of particulate matter in white shrimp is similar to that described for a number of invertebrates but is most closely related to that observed in insects. The first observed event of the internal defense mechanism of the white shrimp in response to the injected carmine particles was possibly due to a humoral factor of the hemolymph which caused the particles to adhere together forming extracellular clumps. Next, the clumps were infiltrated and the particles phagocytized by hemocytes and wandering hematogenous cells. Many of these cells

then returned to the general circulation and migrated to the external surface through the epithelia of the gills, gut and hepatopancreas. Hemocytes containing carmine may also have been eliminated via the antennule gland and the terminal ampoule of the male. Congestion of the muscle and loose connective tissue of the maxillae, pereiopods, and pleopods with hemocytes that eventually migrate to the external surface, apparently through the cuticular pores at the basis of the setae, is also proposed as an important route of elimination. The secondary phagocytosis by fixed phagocytes in the loose connective tissue and by phagocytic cells lining the hemolymph sinuses of the heart, gill, and abdomen is a probable explanation for the apparent slow clearance rate of the carmine particles from white shrimp.

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